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Multiple system atrophy (MSA) is a progressive neurological disorder characterized by parkinsonism, cerebellar dysfunction and autonomic impairment. The cardinal pathological feature of MSA is the glial cytoplasmic inclusion (GCI) in oligodendrocytes, and a major component of GCIs is α-synuclein. We identified a rat oligodendrocytic precursor cell line (CG-4) that produces α-synuclein and characterized α-synuclein in the cells and production of α-synuclein after differentiation to mature oligodendrocytes and astrocytes. Studies suggest that oxidative stress can cause aggregation of α-synuclein. We have further developed CG-4 cells that can overexpress human α-synuclein constitutively or under the control of a tetracycline inducible promoter and have characterized these cells. Overexpression of human α-synuclein appears to make to cells more vulnerable to oxidative stress.

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Journal articles

Tsuboi K, Hashimoto M, Masliah E, Shults CW.
Expression of α-synuclein immunoreactive in oligodendrocyte progenitor cells.
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Meeting abstracts

Tsuboi K, Hashimoto M, Masliah E, Shults CW.
Alpha-synuclein immunoreactive materials in oligodendrocytic progenitor cells.
Program No. 689.3. 2002 Abstract Viewer/Itinerary Planner. Wshington, DC: Society for Neuroscience, 2002. Online.

Shults CW, Hashimoto M, Masliah E, Tsuboi K.
Stable human α-synuclein overexpressing oligodendrocytic progenitor cells.
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INTRODUCTION

Multiple system atrophy (MSA) is a progressive neurological disorder characterized by parkinsonism, cerebellar dysfunction and autonomic impairment. The cardinal pathological feature of MSA is the glial cytoplasmic inclusion (GCI) in oligodendrocytes, and a major component of GCIs is α-synuclein. We identified an oligodendrocytic precursor cell line, CG-4, that endogenously produces α-synuclein. We characterized α-synuclein-immunoreactive (α-synuclein-IR) material in the CG-4 cells (with attention to higher molecular weight forms), and studied the effects of oxidative stress. We also produced CG-4 cells that constitutively overexpress human α-synuclein and CG-4 cells that express human α-synuclein under control of the tetracycline-ON (inducible promoter) system, and we have characterized both of these.

BODY

1. Characterization of CG-4 Cells

Maintenance and differentiation of the cells - CG-4 cells were kindly provided by Dr. R. H. Quarles (NIH). The cells were maintained in cell growth medium consisting of 70% Dulbecco's modified Eagle's media with N-1 supplement (DMEM-N1media) plus 10 ng/ml biotin and 30% B104 conditioned media (Louis et al., 1992). To differentiate the CG-4 cells into oligodendrocytes, the cells were washed twice with Hank's balanced salt solution (HBSS) and maintained in DMEM-N1 media plus 10ng/ml biotin for 6 days. After 48 hr, 0.1% fetal calf serum was added to the media to promote survival of differentiated oligodendrocytes. To differentiate CG-4 cells into astrocytes, CG-4 cells were washed twice with HBSS and 20% fetal calf serum was added to the DMEM-N1 media. Stable human α-synuclein transfected B103 cells and vector transfected B103 cells were used as control cells (Takenouchi et al., 2001).

Immunohistochemistry - Cells were seeded onto poly-L-ornithine coated coverslips and maintained under undifferentiated condition or differentiated condition, as previously described. The cells were fixed with 4% paraformaldehyde for 20 min. After three rinses in PBS, the cells were incubated in PBS/0.25% Triton X-100/20% normal goat serum for 1 hr. For single-labeling, the cells were incubated in monoclonal anti-synuclein-1 (1:100, Transduction Lab.) overnight at 4°C. After washing with PBS/0.25% Triton X-100 three times, the cells were incubated in biotin-conjugated anti-mouse IgG (Jackson Immunoresearch Lab.) for 1 hr and rinsed. The cells were then incubated with Cy3-streptavidin (Jackson ImmunoResearch Lab) for 1 hr. The coverslips were washed three times in PBS and mounted.

For double-labeling, the cells were first incubated in cell specific antisera, either anti-A2B5 (1:200, Chemicon), anti-myelin basic protein (MBP) (1:800, a gift of Dr. John Whitaker), or anti-glial fibrillary acidic protein (GFAP) (1:200, Chemicon) for 1 hr. After washing, the cells were incubated in the corresponding secondary antibodies, FITC-conjugated anti-mouse IgM (Jackson ImmunoRes Lab) for anti-A2B5, FITC-conjugated

anti-rabbit IgG (1:100, Jackson ImmunoRes Lab) for anti-MBP or anti-GFAP, for 1 hr. Anti-synuclein 1 staining described above was followed after washing with PBS/0.25% Triton X-100 three times.

Preparation of cell lysate - Cells were washed with PBS twice and lysed with ice-cold lysis buffer (1 mM Hepes, 5 mM benzamidine, 2 mM 2-mercaptoethanol, 3 mM EDTA, 0.5mM magnesium sulfate, 0.05% sodium azide and 10 μg/ml leupeptin). The cells were sonicated using a ultrasonic processor three times for 5 sec on ice. The cell lysate was separated by centrifugation for at 100,000 x g at 4°C for 1 hr into supernatants (cytosolic fraction) and pellets (particulate fraction). The pellets were resuspended with the lysis buffer described above. Protein content in both cytosolic and particulate fraction was determined by BCA protein assay (Sigma).

Immunoblotting - Proteins were separated by SDS-PAGE, and transferred onto nitrocellulose membranes as previously described (Hashimoto et al., 1999). The blots were blocked with 3% BSA in Tris buffered saline (TBS) containing 0.2% Tween (T-TBS) and NP-40 for 30 min. After blocking, the blots were incubated overnight at 4°C with monoclonal anti-α-synuclein antibody (1:1000, Transduction Lab). The blots were washes with T-TBS containing 0.1% NP-40 three times and incubated with peroxidase conjugated anti-mouse IgG (1:10,000, JacksonImmuno.) for 1.5 hr. After washing three times with T-TBS containing 0.1% NP-40, immunoreactive bands were detected with enhanced chemiluminescence (Amersham).

Two-dimensional gel electrophoresis - Duplicate two-dimensional electrophoresis was performed by Kendrick Labs Inc (Madison, WI) as follows: Isoelectoric focusing was carried out in glass tubes of inner diameter 2.0 mm using 2% pH 3.5-10 (Amersham PharmaciaBiotech). One μg of an isoelectric focusing internal standard, tropomyosin, was added to the sample. After equilibration for 10 min in Buffer'O' (10% glycerol, 50 mM dithiothreitol, 2.3% SDD and 62.5 mM Tris, pH 6.8), the tube gels were sealed to the top of the stacking gel of 10% acrylamide Tris-HCl gel and SDS-PAGE was carried out.

Mass Spectrometry analysis - One of the duplicate gels was stained with Coomassie Blue and the other one was transferred to PVDF membrane. The blot was stained with Coomassie and scanned on a desk-top scanner. Then the blot was immunoblotted with anti-α-synuclein (Transduction Lab) as described before. Coomassie blue-stained spot was cut out from 2D gel corresponding the immunoblot and digested with trypsin. The resulting peptide mixture was subjected to MALDI-MS at the Protein Chemistry Core Facility at Howard Hughes Medical Institute (Columbia University, New York, NY).

Alpha-synuclein endogenous expression in CG-4 - In order to investigate the expression of α-synuclein in CG-4 cells, cytosolic fraction and particulate fractions of CG-4 cells were compared with those of human α-synuclein transfected B103 and vector transfected B103 described previously (Takenouchi et al., 2001). In CG-4 cells, endogenous α-synuclein-IR material, which migrated with our positive controls (rat brain homogenate and recombinant human α-synuclein and rat brain homogenate), was

detected in the cytosolic fraction cells, while it was barely detected in vector transfected B103. However, the amount of α-synuclein-IR material was rather moderate when compared with that of human α-synuclein transfected B103. In all three cell lines, intense 46kD α-synuclein-IR material was observed in both cytosolic and particulate fractions. 33kD α-synuclein-IR material in the particulate fraction distinguished CG-4 cells from vector transfected B103. Further analysis of α-synuclein expression and distribution of CG-4 cells were performed by immunolabelling. Alpha-synuclein-IR materials were diffusely distributed in cell bodies of CG4 cells. Alpha-synuclein immunoreactivity was more abundantly observed in human α-synuclein transfected B103 but not in vector transfected B103. No synuclein-IR inclusions were observed in any cells. Control experiments were carried out by omitting the primary antibodies, and faint, nonspecific labeling was observed.

Characterization of high MW of α-synuclein-IR material in CG4 - To characterize the 46kD α-synuclein immunoreactive band, duplicate 2D SDS-PAGE were performed, followed by Coomassie blue staining or immunoblotting. The 46kD kD α-synuclein immunoreactive material had a basic pI, while 20kD α-synuclein immunoreactive material had an acidic pI. Using MALDI-MS, the proteins in the Commassie-stained spot corresponding to 49kD α-synuclein immunoreactive material were analyzed. We identified the mixture of β-tubulin (Accession No. P04691), γ-enolase (Accession No. P07323), and AK003217 (Accession No. 12833747) in this region. AK003217 is a putative compound that was identified by cDNA cloning studies from 18-day-old mouse embryo and its function is not known yet (Kawai et al., 2001). There was no distinct Coomassie-stained spot corresponding to 20kD α-synuclein immunoreactive material. We further investigated the possibility that the proteins identified in the 46kD α-synuclein band (β-tubulin, γ-enolase, and AK003217) cross-react with anti-synuclein-1 antibody. Both γ-enolase and β-tubulin migrated at 46kD in 1D SDS-PAGE (data not shown). Beta-tubulin (100 ng - 10 μg) did not cross-react with anti-synuclein-1. Unexpectedly, 20kD synuclein-1 immunoreactive material was noted in the immunoblot of γ-enolase using the anti-synuclein-1 antibody, suggesting that the purified γ-enolase contained α-synuclein monomer (data not shown). The intensity of 46kD synuclein-1 immunoreactive material in γ-enolase (2.5 μg) was much weaker than the intensity of 46kD synuclein-1 immunoreactive material in CG-4 (total 25 μg protein), indicating that the immunoreactivity could not be fully attributed to cross reactivity with γ-enolase (data not shown). AK003217 was not tested due to unavailability.

Alpha-synuclein expression after differentiation to oligodendrocytes - To investigate the expression of α-synuclein in CG-4 cells after differentiation, CG-4 cells were differentiated into an oligodendrocytic phenotype or an astrocytic phenotype and compared with CG-4 cells in undifferentiated condition. Most of CG-4 cells in undifferentiated condition were labeled with the O-2A progenitor marker, anti-A2B5. Alpha-synuclein was diffusely distributed in the cytoplasm of A2B5-positive cell. After differentiation into an oligodendrocytic phenotype, we confirmed that CG-4 cells were labeled with mature oligodendrocyte marker, anti-MBP. After differentiation into astrocytic phenotype, we confirmed that CG-4 cells were labeled with the astrocytic

marker, GFAP. In GFAP-IR CG-4 cells, α-synuclein was diffusely distributed in the cell bodies.

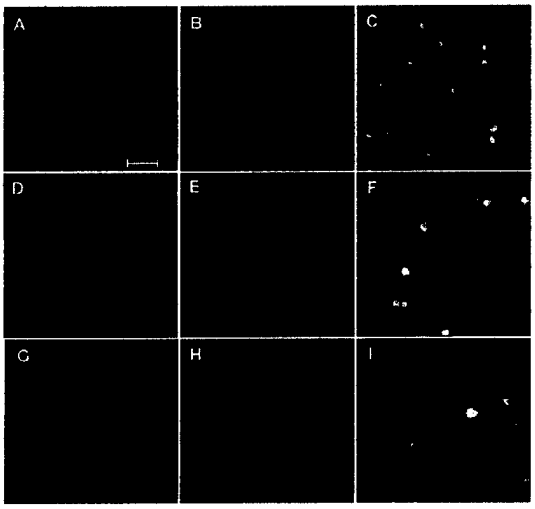


Fig. 1. Double-labeling with cell markers (green) and anti-synuclein 1 (red) in CG-4 cells at three differentiating conditions, undifferentiated CG-4 (A-C), CG-4 cells after differentiating into an oligodendrocytic phenotype (D-F), and CG-4 cells after differentiating into an astrocytic phenotype (G-I). A, anti-A2B5, oligodendrocyte/astrocyte progenitor marker; D, anti-myelin basic protein, mature oligodendrocyte marker; G, anti-glial fibrillary acidic protein, astrocyte maker; B, E and H, anti-synuclein 1 labeling; C, F, and I, merged images. Scale bar indicates 50 μm. (See appendix for larger figure)

To compare the expression of α-synuclein in CG-4 after differentiation, we examined three conditions, undifferentiated and differentiated to an oligodendrocytic phenotype (Oligo-differentiated CG-4) or an astrocytic phenotype (Ast-differentiated CG-4). The expression of α-synuclein monomer increased more in Oligo-differentiated CG-4 than in undifferentiated CG-4 and Ast-differentiated, while the expression of 46kD immunoreactive material in the cytosolic fraction decreased more in Oligo-differentiated CG-4 than in the others.

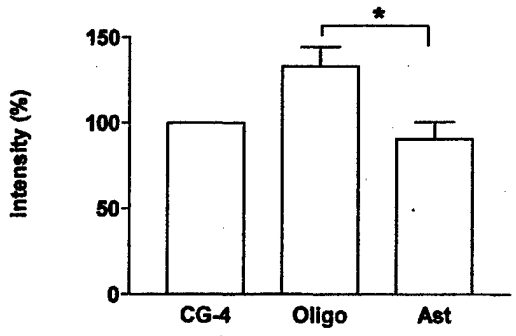
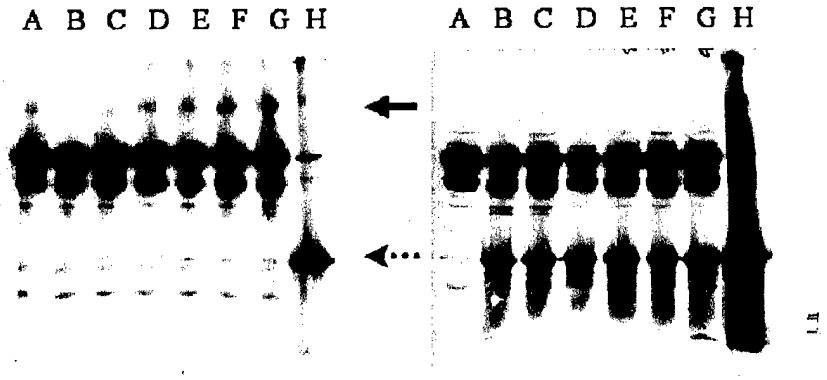


Fig. 2. In parallel CG-4 cells were differentiated to oligodendrocytic (Oigo) or astrocytic (Ast) phenotype or continued undifferentiated. The cells were then harvested and immunoblots performed to identify of α-synuclein monomer, and the density of the bands quantitated and Oligo and Ast were normalized to the value for the CG-4 cells in each of the 4 studies and the means and SEM are presented. Oligo had significantly more of α-synuclein monomer than Ast ($p < 0.05$).

2. Effects of oxidative stress

We have studied the effects of a number of oxidative treatments on aggregation of α-synuclein. To date treatment we have not found that treatment with $FeCl_2$ and H_2O_2 individually caused aggregation.

Fig. 3. In the study illustrated below we noted that treatment of the CG-4 cells with both $FeCl_2$ and H_2O_2 for 48 hours increased the aggregation of α-synuclein. Immunoblots of the particulate fraction (left) and cytosolic fraction (right) fractions from cell lysates cells were labeled with antibody (Transduction) raised against rat α-synuclein: lane A - B104 neuroblastoma cells; lanes B-G - CG-4 cells; lane H - rat brain homogenate. The CG-4 cells had been treated for 48 hours with 200 μM $FeCl_2$ (lanes C-G) and H_2O_2 (lanes D-100 μM, E-200 μM, F-300 μM, G-400 μM). The dashed arrow indicates α-synuclein monomer. The solid arrow indicates a α-synuclein-IR material at a higher molecular weight, which increases with increasing concentration of H_2O_2 and is consistent with aggregation of α-synuclein.



In normal CG-4 cells, we have not found that treatment with the nitration agent S-nitrosylglutathione or with the proteasome inhibitor lactacystin resulted in obvious aggregation.

3. Constitutive overexpression of human α-synuclein in CG-4

CG-4 cells were maintained in cell growth medium consisting of 70% Dulbecco's modified Eagle's media with N-1 supplement (DMEM-N1media) plus 10 ng/ml biotin and 30% B104 conditioned media. *cDNA subcloning* - pLNCX2 retroviral vector and hα-synuclein cDNA were digested with appropriate restriction enzymes (NotI and SalI) and purified after treating with phosphatase. The digested vector and hα-synuclein gene fragment were ligated and transformed into E. coli. Alpha-synuclein-pLNCX2 retroviral vector was identified by restriction analysis. *Virus production* - EcoPack 2^{TM}-293 packaging cells were seeded in 100 mm tissue culture and allowed to grow 70% confluence. Ten μg of plasmid DNA (pLNCX2-α-synuclein- retroviral vector or insert

free pLNCX2 retroviral vector) was used to transfect cells in 100-mm plate, using Superfect (Qiagen). Culture medium was aspirated 2.5 hr after transfection, and 10 ml of complete medium was added. To increase viral titer, cultures were incubated for 48 hr.

Virus infection-CG-4 cells were plated at a cell density of 1-2 x 10^5 per 60-mm plate 12-18 hr before infection. Medium from packaging cells was collected and filtered through a 0.45-μm cellulose acetate or polysulfonic filters. Virus was added to target cells, and polybrene was added to culture medium. Medium was replaced with fresh medium after 24 hr of incubation. The infected cells were selected with G418 48 hours after infection. Large, healthy colonies were isolated using cloning cylinders and were maintained. Twenty-one colonies were screened by immunoblotting and immunolabelling.

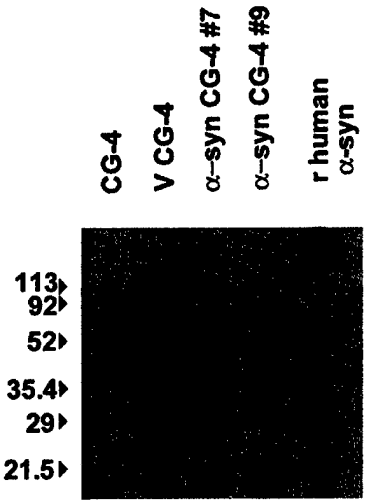


Fig 4. Immunoblots of the cytosolic fractions from CG-4 cells (CG-4), CG-4 cells that had been infected with vector only (V CG-4), CG-4 cells that that been infected with the vector containing the cDNA for hα-synuclein (CG-4#7 and CG-4#9) and recombinant hα-synuclein. The immunoblots were labeled with the antibody LB509, which specifically recognizes hα-synuclein. In the cytosolic fractions, only the CG-4 cells infected with vector containing hα-synuclein cDNA produced hα-synuclein, and the amount expressed was greater in CG-4#9 than in CG-4#7 and the total amount of α-synuclein (rat + human) greatly exceeds the amount of rat α-synuclein found normally in the wild type CG-4 cells (data not shown).

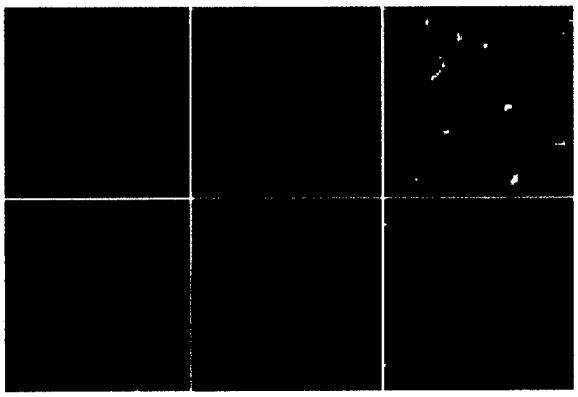


Fig 5. Cell lines CG-4#9 (top row) and V CG-4 (bottom row) were immunolabelled for the marker of oligodendrocytic precursor cells A2B5 (left, green image) and for hα-synuclein (center, red image). Merged images (right, yellow for colocalized images) indicated that most, but not all, of the CG-4#9 cells produced hα-synuclein. V CG-4 cells had only faint, non-specific immunolabelling for hα-synuclein. (See appendix for larger image).

4. Expression of human α-synuclein under control of the tetracycline-inducible promoter

EcoPack 2 packaging cells were transfected with pRevTet-On vector, pRevTRE-hα-synuclein vector or pRevTRE-Luc vector, as described above. Virus containing media were collected 48 hr later and filtered with 0.45 μm filter. CG-4 cells were infected with virus-containing medium (RevTet-On) as described above. Two days after infection, the cells were treated with G418. Once colonies were visible, healthy colonies were isolated and transferred to individual wells. To identify G418 resistant clones that have high inducibility and low background, each clone was infected with RevTRE-Luc. Luciferase assay was performed to identify appropriate clones. Clones with highest fold-induction were selected and expanded.

Stable Tet-On CG4 cells were infected with RevTRE-hα-synuclein virus, as described above. After selecting clones with hygromycin (pRevTRE-hα-synuclein also carries the gene for hygromycin resistance), 33 clones were isolated. We have screened the clones for production of hα-synuclein after exposure to doxycycline and 7 of 28 have shown the expression of hα-synuclein is expressed under the control of the Tet-ON system.

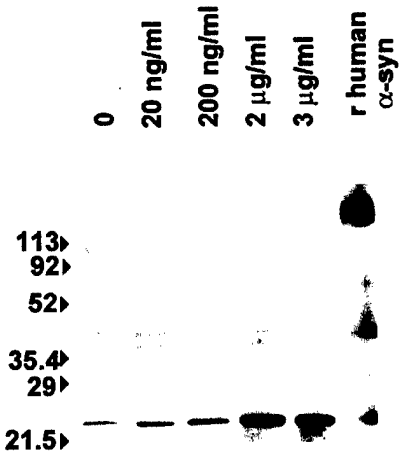


Fig 6. Immunoblots of the cytosolic fraction from cell line 9-50-8, which had been genetically engineered to express hα-synuclein under the control of the Tet-ON system, after exposure to doxycycline for 48 hours with 0, 20 ng/ml, 200 ng/ml, 2 μg/ml or 3 μg/ml of doxycycline. Immunoblots were performed using antibody LB509, which specifically recognizes hα-synuclein. The amount of hα-synuclein monomer increased in the cultures with greater dose of doxycycline, but there was no clear increase above the 2 μg/ml dose. Doxycycline is not toxic to CG-4 cells within the doses tested.

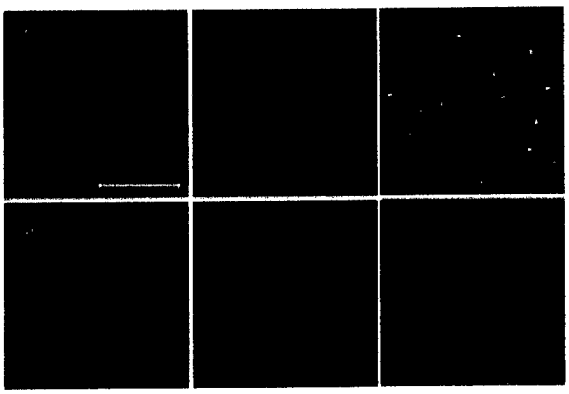


Fig 7. Cell line 9-50-8 after incubation with (top row) or without (bottom row) doxycycline (2 μg/ml, 48 hours) were immunolabelled for the marker of oligodendrocytic precursor cells A2B5 (left, green) and for hα-synuclein (center, red). Colocalization is shown in merged images (right, yellow). Many but not all of the cells produced hα-synuclein after exposure to doxycycline (2 μg/ml, 48 hours). Without exposure to doxycycline, no significant expression of hα-synuclein was noted. (See appendix for larger figure)

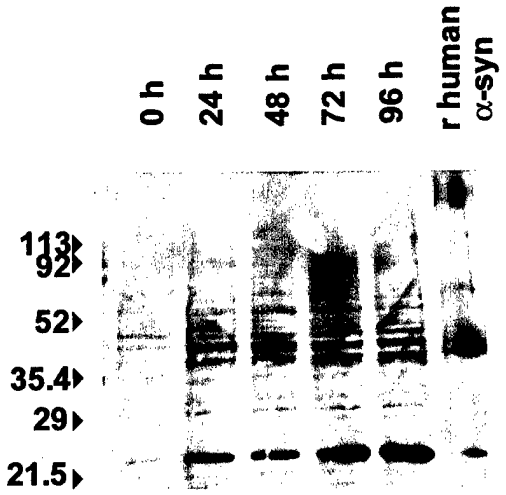


Fig 8. Immunoblots of the cytosolic fraction from cell line 9-50-8, which had been genetically engineered to express hα-synuclein under the control of the Tet-ON system, after exposure to doxycycline (2 μg/ml) for various times (0, 24, 48, 72, 96 hours). Immunoblots were performed using antibody LB509, which specifically recognizes hα-synuclein. The amount of hα-synuclein monomer increased in the cultures with increasing time of exposure to doxycycline.

To evaluate the effects of hα-synuclein overexpression on the toxicity of H_2O_2 we utilized cell line 9-50-8, which overexpresses hα-synuclein under the control of the Tet-ON system, and propidium iodide labeling and flow cytometry to differentiate intact cells versus damaged or dead cells, which could no longer exclude the dye (Davey et al., 1999; Davies et al, 2000).

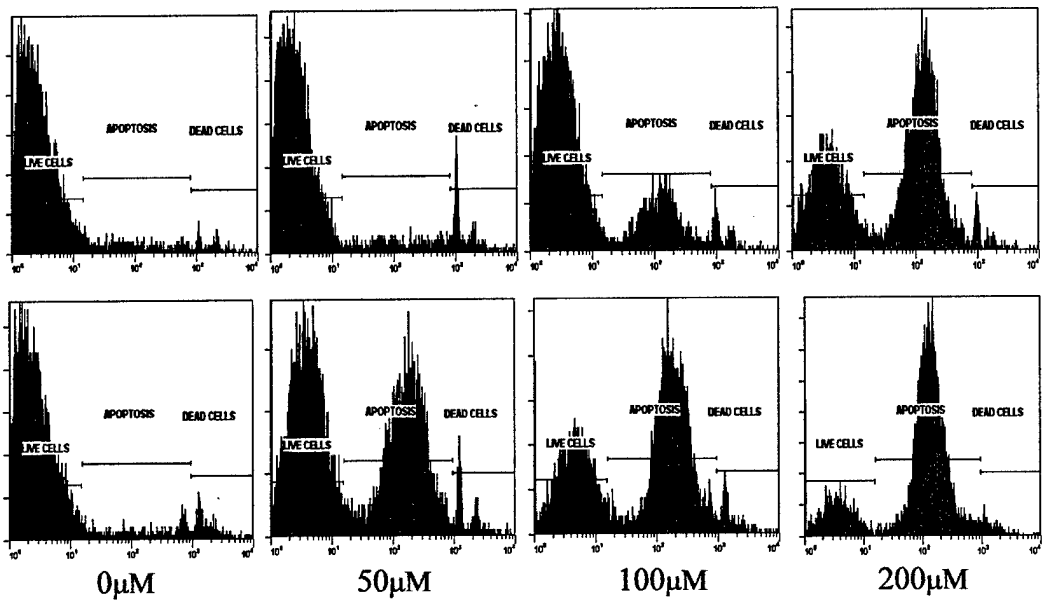


Fig. 9. Cell line 9-50-8 was treated with 2 μg/ml doxycycline (lower panel) or without doxycycline (upper panel) for 48 hrs and the cells were treated with H_2O_2 (0 – 200 μM) for 24 hrs. The cells were harvested and centrifuged at 2,800 rpm for 5 min. The cell pellets were resuspended with 0.5 ml media. Twenty μl propidium iodide (50 μg/ml) was added to each cell suspension. After 10 min, a total of 10,000 cells were analyzed in each case by flow cytometry with Beckman-Coulter Epics Elite (software: Expo 32) with excitation at 488 nm and emission reading at 675 nm. Y axis represents the number of cells with the maximum adjusted to the maximum for each study, typically 50-60. X axis represents the intensity of fluorescence (10^0 to 10^4). Both cells in the "dead cells" range, which had the greatest accumulation of propidium iodide, and the cells in "apoptosis" range, which were unable to fully exclude the dye, were not considered "live cells." Overexpression of hα-synuclein increased the vulnerability to H_2O_2.

KEY RESEARCH ACCOMPLISHMENTS

1. Characterization of α-synuclein-IR material in an oligodendrocytic cell line (CG-4). This thorough characterization will allow investigators in MSA research to utilize the CG-4 cell line for research in MSA.

2. To date our research suggests that oxidative stress can cause aggregation of α-synuclein in oligodendrocytic cells. We are in the process of extending these results in cells that overexpress human α-synuclein.

3. Production of CG-4 cells that overexpress h α-synuclein constitutively or express it in a controlled fashion under the control of the Tet-ON system.

4. Our research indicates that overexpression of hα-synuclein increases vulnerability to oxidative stress.

REPORTABLE OUTCOMES

The above work was presented at the 2002 Annual Meeting of the Society for Neuroscience in two presentations.

Tsuboi K, Hashimoto M, Masliah E, Shults CW. Alpha-synuclein immunoreactive materials in oligodendrocytic progenitor cells.

Shults CW, Hashimoto M, Masliah E, Tsuboi K. Stable human alpha-synuclein overexpressing oligodendrocytic progenitor cells.

Manuscripts are being prepared from the work described in these abstracts.

CONCLUSIONS

Alpha-synuclein occurs in an oligodendrocytic precursor cell line and persists when the cells are differentiated to oligodendrocytes and astrocytes. Under certain conditions, oxidative stress can favor aggregation of α-synuclein in oligodendrocytic cells. It is possible to develop oligodendrocytic cells that overexpress α-synuclein both constitutively and under the control of the Tet-ON system, and these cells appear more vulnerable to oxidative stress.

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APPENDIX COVER SHEET

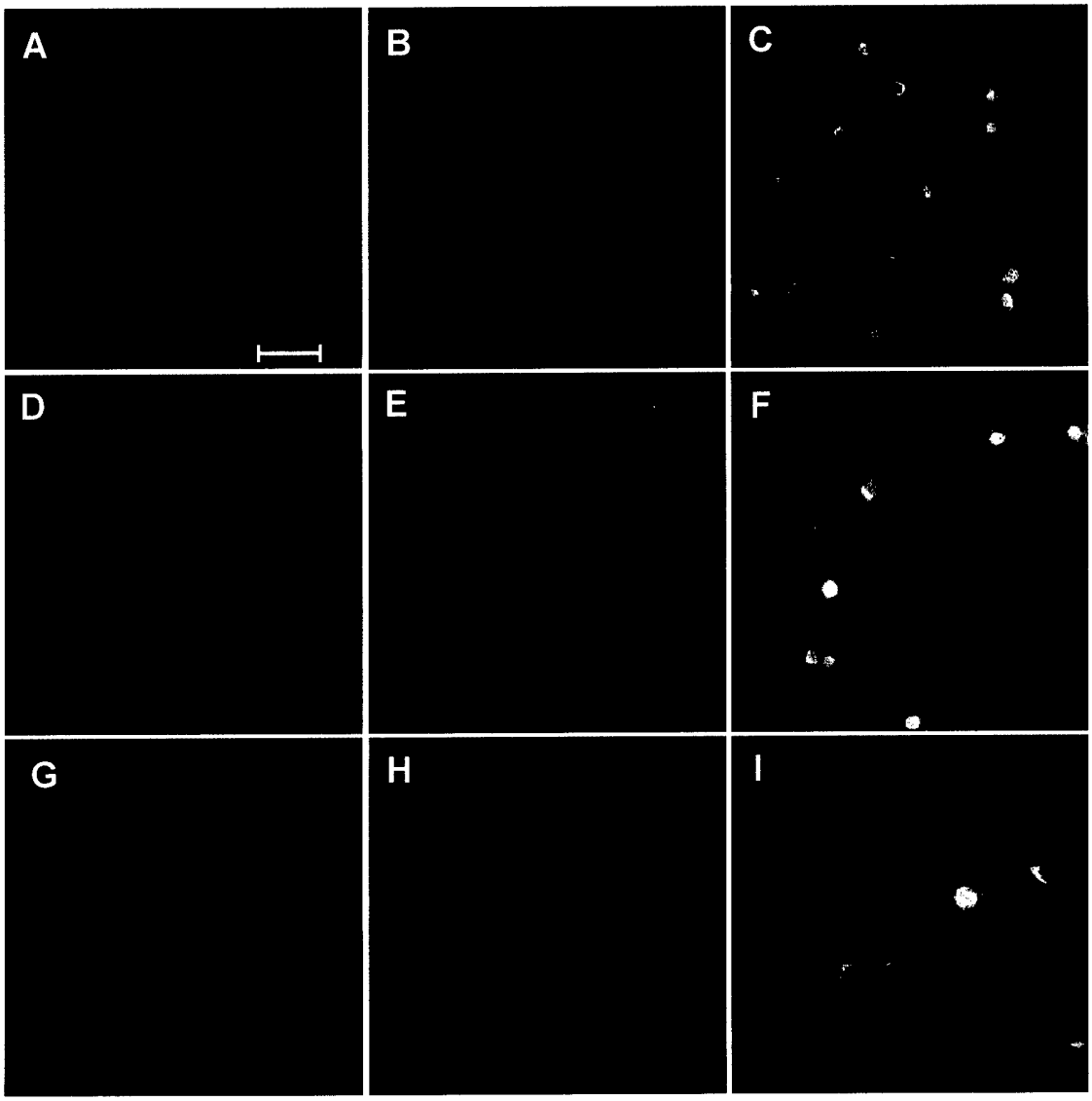

Fig.1

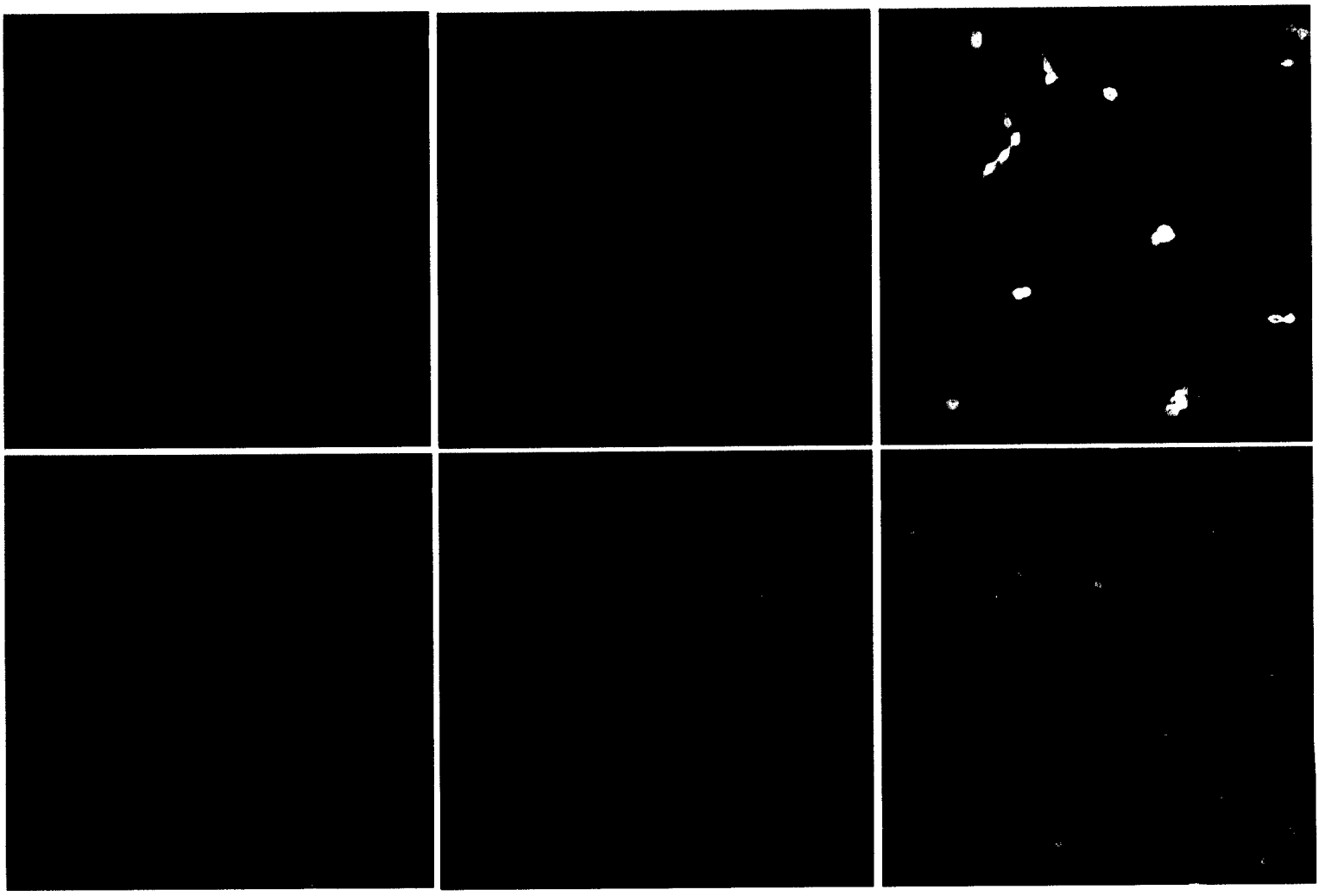

Fig. 5

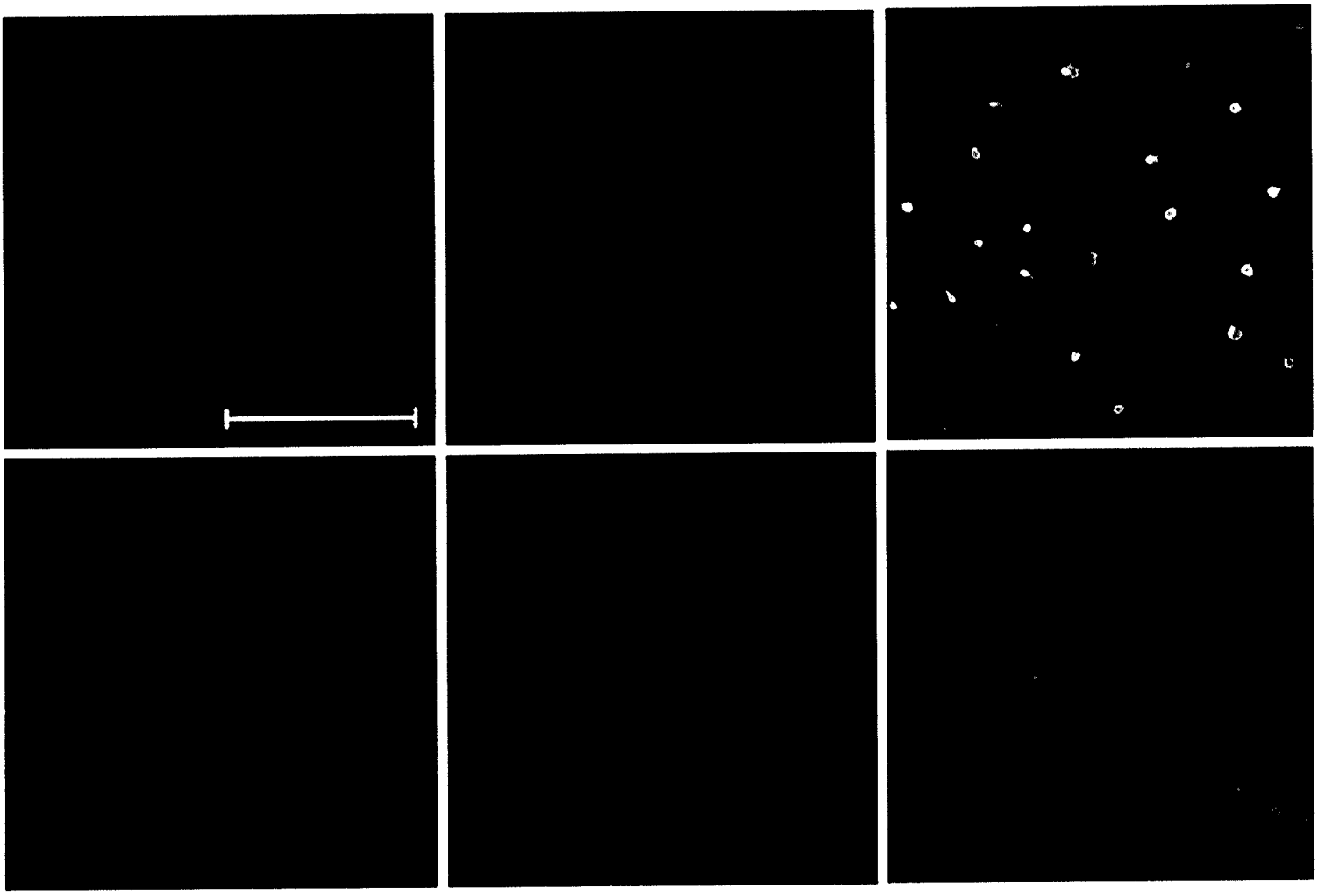

Fig. 7

Abstract View

STABLE HUMAN ALPHA-SYNUCLEIN OVEREXPRESSING OLIGODENDROCYTIC PROGENITOR CELLS

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Multiple system atrophy (MSA) is a progressive, degenerative neurological disorder characterized by parkinsonism, ataxia and autonomic dysfunction. The cardinal pathological feature of MSA is the presence of glial cytoplasmic inclusions (GCIs), in which α-synuclein is a major constituent. Although α-synuclein is primarily a neuronal protein, which accumulates in the synapses and may play a role in plasticity, recent studies suggest that low levels of α-synuclein are present is oligodendrocytes. Since mechanisms for formation of GCIs in MSA are not clear, we undertook to produce an oligodendrocytic cell line overexpressing human α-synuclein. Rat CG-4 cell line was selected because of its normal complement of chromosomes and its capability to differentiate to oligodendrocytes and astrocytes. Rat CG-4 cells were infected with human α-synuclein inserted retrovirus or insert-free (control) retrovirus and selected with G418. Western blot and immunohistochemistry with human specific α-synuclein antibodies confirmed stable expression of human α-synuclein in the CG-4 cell line. This cell line will be a useful model to clarify the mechanisms underlying MSA.

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